^{13}C-egg white breath test: a non-invasive test of pancreatic trypsin activity in the small intestine

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Abstract

Background—The recent availability of egg white protein highly enriched with ^{13}C has allowed breath test technology to be adapted for the study of protein digestion and absorption. Pancreatic trypsin is considered to be the key enzyme in the proteolytic cascade.

Aim—To evaluate trypsin activity in the small intestine of healthy volunteers and patients with pancreatic disease by a recently developed ^{13}C-egg white breath test.

Methods—A total of 48 healthy volunteers and 30 patients with pancreatic disease were studied after ingestion of a test meal consisting of 22 g ^{13}C-labelled egg protein. Breath samples were taken before and after ingestion of the meal and analysed for $^{13}CO_2$ concentration. Moreover, pancreatic trypsin output after maximal stimulation was measured in 13 patients and nine healthy volunteers.

Results—The six hour cumulative $^{13}CO_2$ excretion in breath was significantly lower in patients than controls (mean (SEM): 6.23 (0.82)% *v* 19.16 (0.58)%, p<0.0001). An excellent correlation was found between the six hour cumulative $^{13}CO_2$ excretion and trypsin activity after maximal pancreatic stimulation.

Conclusion—The non-invasive ^{13}C-egg white breath test is promising as an indirect pancreatic proteolytic function test.

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Dietary protein provides 10–15% of the total energy intake of people on a standard Western diet and supplies the essential amino acids required for protein synthesis. The nutritional value of dietary proteins depends both on the concentration and distribution pattern of their constituent amino acids and on their bioavailability—that is, the proportion of amino acids available for metabolic utilisation.[1] The bioavailability of protein is determined by digestive and absorptive events in the upper gastrointestinal tract. Protein digestion implies a complex series of degradative processes which are elicited by the hydrolytic enzymes originating from the stomach, pancreas, and the brush border of the small intestine.[2–4] The result of this proteolytic activity is a mixture of amino acids and small peptides which are absorbed by the small intestinal enterocytes.

Pancreatic digestion most probably plays a critical role in overall protein assimilation. Based on studies comparing duodenal trypsin output with faecal nitrogen output, it was concluded that the exocrine pancreas has a large reserve capacity for protease secretion. It was shown that creatorrhoea (>2.5 g of stool nitrogen per 24 hours) only occurs when trypsin output is less than 10% of normal.[5] These studies, however, assume that the faecal nitrogen output accurately reflects the efficiency of protein digestion and absorption in the small bowel. This is not necessarily true, as nitrogen may be absorbed from and secreted into the colonic lumen to a varying extent.[2 6 7] Ileal intubation experiments have been designed to circumvent the problem presented by colonic nitrogen transport.[8 9] Ileal intubation techniques are, however, time consuming and invasive and moreover appear to delay gastric emptying and to shorten transit time in the small intestine.[10] Shortening of the transit time may give rise to impaired assimilation of macronutrients.[11 12]

Very recently, we succeeded in producing egg protein labelled with stable isotopes in an easily reproducible and efficient manner.[13] The availability of protein highly enriched with ^{13}C allowed breath test technology to be adapted for the study of protein assimilation. Breath tests, in general, are based on the administration of a substrate with a functional group containing a carbon atom with either the radioactive (^{14}C) or the stable (^{13}C) isotope of carbon. The functional group is enzymatically cleaved during passage through the gastrointestinal tract, during its absorption, or in subsequent metabolic processes. After cleavage of the target bond, the cleaved portion undergoes further metabolism to $^{14}CO_2$ or $^{13}CO_2$, which mixes with the bicarbonate pool of the blood and is finally expired in the breath. In this way, the $^{14/13}CO_2$ excretion is a reflection of the total amount or kinetic properties of the enzyme studied, given that this enzyme relates to the rate limiting step in the whole process. Breath tests are simple, easily repeatable, and non-invasive. They are moreover very interesting as a functional test because the information obtained represents a dynamic evaluation rather than a static estimation.[14 15]

The aim of this study was to evaluate trypsin activity in the small intestine of healthy volunteers and patients with pancreatic disease by a recently developed ^{13}C-egg white breath test.

Materials and methods

PATIENTS AND CONTROLS

Thirty patients with pancreatic disease were studied (five women and 25 men, aged 34–70).

Table 1   *Characteristics of patients with pancreatic disease (n = 30)*

No	Sex	Disease	Cause	Pancreatin	Surgery	CAT scan	ERP*	GT†	% dose ¹³C cum
1	M	CP	Ethanol	No		Atrophic pancreas, dilatation of pancreatic ducts		1	3.81
2	M	CP	Unknown	1.8 g/day		Pancreas divisum, dilatation of duct of Wirsung	4	1	5.75
3	M	CP	Ethanol	1.8 g/day		Dilatation of the duct of Wirsung	4	1	16.46
4	M	CCP	Ethanol	7.2 g/day	Subtotal pancreatectomy	Calcifications		3	5.29
5	M	CCP	Ethanol	No		Calcifications	3	1	8.61
6	V	CCP	Ethanol	No		Calcifications, atrophic pancreas	5	1	1.20
7	M	CCP	Ethanol	3.6 g/day		Calcifications	4	3	13.63
8	V	CCP	Ethanol	No		Calcifications, pseudocyst	5	1	9.48
9	V	CCP	Ethanol	2.7 g/day	Subtotal pancreatectomy	Calcifications		3	5.88
10	M	CCP	Ethanol	3.6 g/day	Subtotal pancreatectomy	Calcifications		3	2.77
11	V	CCP	Ethanol	Unknown		Calcifications, pseudocysts	3	1	7.55
12	M	PCa		3.6 g/day	Subtotal pancreatectomy			3	0.53
13	M	PCa		No	Subtotal pancreatectomy			3	0.00
14	M	CCP	Ethanol	1.8 g/day		Calcifications, pseudocysts		1	9.15
15	M	CCP	Ethanol	Unknown		Calcifications, dilatation of duct of Wirsung, pseudocysts		1	9.53
16	M	CCP	Ethanol	No		Calcifications, pseudocysts	5	1	5.06
17	M	CCP	Ethanol	5.4 g/day		Calcifications	4	3	0.24
18	M	CCP	Unknown	Unknown	Subtotal pancreatectomy	Calcifications		2	7.64
19	M	CCP	Unknown	2.7 g/day		Calcifications, pseudocysts		1	1.48
20	M	CCP	Ethanol	No		Calcifications	3	1	6.50
21	M	PCa		0.6 g/day	Total pancreatectomy			3	0.00
22	M	CCP	Ethanol	2.7 g/day		Calcifications		3	7.17
23	M	CCP	Unknown	1.8 g/day		Calcifications	3	3	11.23
24	V	CCP	Ethanol	1.8 g/day		Atrophic pancreas, calcifications	4	1	6.76
25	M	CP	Ethanol	Variable	Subtotal pancreatectomy		3	1	12.93
26	M	CP	Unknown	No	Bilroth II gastrectomy		3	1	5.40
27	M	CCP	Ethanol	1.2 g/day		Calcifications		3	2.94
28	M	CP	Unknown	1.8 g/day		Atrophic pancreas		1	14.12
29	M	CCP	Ethanol	No		Atrophic pancreas, calcifications		1	4.89
30	M	CCP	Ethanol	7.2 g/day	Subtotal pancreatectomy	Calcifications		3	0.82

CAT, computed axial tomography; ERP, endoscopic retrograde pancreatography; GT, glucose tolerance; % dose ¹³C cum, percentage of administered dose of ¹³C recovered in breath over six hours; P, chronic calcifying pancreatitis; CP, chronic pancreatitis; PCa, pancreatic carcinoma; *ERP findings: 3 = moderate pancreatitis; 4 = advanced change pancreatitis (ACP), with dilated ducts, 5 = ACP with obstructed duct. †Glucose tolerance: 1 = normal, 2 = glucose intolerance by oral glucose tolerance test, 3 = overt diabetes.

Three patients had pancreatic cancer. All of them had undergone pancreatic surgery. Twenty seven patients had chronic pancreatitis; in 21 it was due to alcoholism, and in six no certain cause was identified. The diagnosis of chronic pancreatitis was based on the presence of a typical history, suggestive radiological findings—for example, pancreatic calcifications or pseudocysts—, and/or pathological findings on endoscopic retrograde pancreatography (table 1). Seven patients had undergone pancreatic surgery for complicated chronic pancreatitis.

The mean (SEM) body mass index of the patients was 21.4 (0.8). Small intestinal and hepatobiliary diseases were excluded on the basis of clinical, radiological, and laboratory data. Twelve patients were receiving insulin substitution therapy; 17 were taking pancreatic enzyme supplements. Apart from the insulin substitution therapy, no medication was allowed on the day of the test.

The control group comprised 48 normal subjects (14 women and 34 men; mean age 22 years, range 18–41). The mean (SEM) body mass index of the healthy volunteers was 22.3 (0.4).

The protocol was approved by the ethical committee of the University of Leuven and all subjects gave informed consent.

¹³C-EGG WHITE BREATH TEST
Test meal
The test meal consisted of the yolk of one egg and 22 g of ¹³C-labelled egg white protein. The methodology for obtaining large amounts of highly enriched egg white protein specifically labelled with L-[1-¹³C]leucine has been extensively described elsewhere.[13] Briefly, these proteins were produced by giving laying hens free access to food containing 25% of the National Research Council required leucine content as free L-[1-¹³C]leucine (99 atom%; Euriso-top, Saint-Aubin, France). The egg white of the enriched eggs was separated from the yolk and pooled. The isotopic enrichment was determined using a continuous flow elemental analyser-isotope ratio mass spectrometer (ANCA-SL; Europa Scientific, Crewe, UK). The [1-¹³C]leucine (99 mol%) content of each test meal was calculated separately and taken into account in the calculation procedures. Total energy content of the test meal was 0.627 MJ. It was appropriately baked in a microwave oven. Subjects were urged to consume the test meal together with 200 ml of water within 15 minutes. Sodium chloride was allowed as appetiser.

Breath sampling, analysis, and calculations
All subjects were studied in resting conditions after an overnight fast of at least 12 hours.

Breath samples were collected in exetainers (Europa Scientific) twice immediately before the meal and at 15 minute intervals for a period of six hours after the meal. ¹³C content in breath was determined by on line gas chromatographic purification-isotope ratio mass spectrometry (ABCA; Europa Scientific). The δ values given by the isotope ratio mass spectrometry were converted to percentage ¹³C recovery of the initial amount administered per hour (% dose ¹³C/h) according to calculations described in detail by Ghoos *et al*.[16] Cumulative percentages of label recovery (% dose ¹³C cum) were calculated using the trapezoidal rule. From these data, the following parameters of protein assimilation were derived: the maximum percentage of
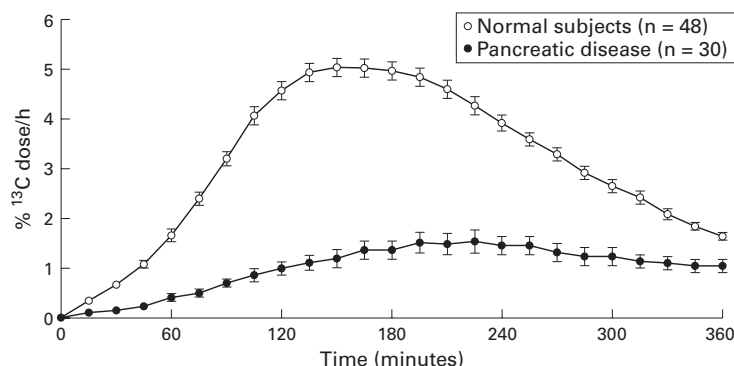
Figure 1   Rate of $^{13}CO_2$ excretion (in percentage of dose per hour) after ingestion of 22 g ^{13}C-labelled egg white protein by normal subjects (n = 48) and patients with pancreatic disease (n = 30). Values are mean (SEM).

administered dose of ^{13}C excreted per hour ($\%_{max}$), the time when $\%_{max}$ is reached (t_{max}), and the cumulative percentage of administered dose of ^{13}C recovered in breath over six hours (% dose ^{13}C cum 6 h).

SERIAL MEASUREMENTS OF PLASMA ^{13}C ENRICHMENT OF LEUCINE

Serial measurements of plasma ^{13}C enrichment of leucine were performed simultaneously with the breath test in a subgroup of six volunteers (two women and four men; mean age 21.7, range 18–24) and two patients (men; mean age 45.5, range 41–50).

Blood sampling, analysis, and calculations

Blood samples were taken via an indwelling intravenous catheter which was placed in the forearm 30 minutes before the start of the test. Blood was sampled before ingestion of the test meal and every 30 minutes thereafter for six hours. The samples were collected in prechilled (4°C) vacutainers containing lithium heparin as anticoagulant (Becton Dickinson Vacutainer Systems Europe, Meylan, France). Plasma was immediately separated in a refrigerated centrifuge and stored at −70°C until analysis. After thawing, the plasma was prepared and derived by the method of Boirie *et al*.[17] The ^{13}C enrichment of leucine was determined by on line gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Briefly, 2 µl of the derived solution was injected into a GC-C-IRMS (Orchid; Europa Scientific). After separation on the analytical CB-Sil 5 MS column (Chrompack, Middelburg, The Netherlands), the derived leucine was combusted on line. The oxidation products were subsequently bled into an isotope ratio mass spectrometer and analysed for $^{13}C/^{12}C$ ratio. The δ values given by the isotope ratio mass spectrometry were converted into mol% excess (MPE) and normalised to an L-[1-^{13}C]leucine intake of 150 mg:

$$MPE_{norm} = MPE \times \frac{150}{\text{amount of [1-}^{13}C]\text{leucine ingested (mg)}}$$

The breath test results of the subjects participating in this part of the study were expressed as atom% excess (APE) and normalised in an analogous manner (APE_{norm}).

MEASUREMENT OF TRYPSIN OUTPUT AFTER MAXIMAL STIMULATION OF THE PANCREAS

The trypsin output was measured by a marker corrected duodenal perfusion technique in 13 of the 30 patients with pancreatic disease and nine of the 48 normal subjects. All 30 patients were invited to participate in this part of the study, but only 13 consented. After an overnight fast, a double lumen duodenal tube and a separate gastric tube were positioned under fluoroscopic control. The perfusion port of the duodenal tube was located near the ampulla of Vater and the aspiration site near the duodenojejunal flexure. Gastric contents were continuously aspirated. A thermally controlled (37°C) mannitol/saline solution containing [^3H]polyethylene glycol 4000 as a non-absorbable marker was infused at a constant rate of 10 ml/min at the proximal duodenal port. After a basal steady state perfusion of 30 minutes, caerulein (0.05 µg/kg body weight) (Takus; Pharmacia, Erlangen, Germany) was injected intravenously over two minutes. The duodenal contents were thereafter collected on ice in four 15 minute intervals. The [^3H]polyethylene glycol content in gastric and duodenal aspirates was measured by the oxidation method (Packard sample oxidiser, model 306; Packard, Downers Grove, Illinois, USA), with subsequent liquid scintillation counting (model 2450; Packard). Trypsin activity was measured spectrophotometrically at 253 nm using *N*-α-benzoyl-L-arginine ethyl ester as substrate.[18] The trypsin output was calculated on the basis of the [^3H]polyethylene glycol recovery data and expressed as units per hour.

STATISTICAL ANALYSIS

Relations between different parameters were calculated by correlation or regression analysis. Differences between sample means were calculated using Student's *t* test. All values are expressed as mean (SEM).

Results

$^{13}CO_2$ BREATH TESTS

Figure 1 gives the course of the ^{13}C excretion rate, expressed as % dose/h, in normal subjects after ingestion of the ^{13}C-labelled egg protein meal. The $\%_{max}$ amounted to 5.61 (0.17)%/h and was reached 165 (6) min after the protein meal was ingested. The course of the $^{13}CO_2$ excretion rate was appreciably different in patients with pancreatic disease; the $^{13}CO_2$ excretion rate rose much more slowly (t_{max} 220 (16) minutes), did not reach high values ($\%_{max}$ 1.92 (0.22)%/h), and remained on a rather constant level after maximal excretion was reached. The differences between normal subjects and patients were highly significant ($\%_{max}$, $p < 0.0001$; t_{max}, $p < 0.005$).

The mean % dose ^{13}C cum 6h amounted to 19.16 (0.58)% in normal subjects and 6.23% (0.82)% in patients with pancreatic disease. The differences between normal subjects and patients were highly significant (% dose ^{13}C cum 6h: $p < 0.0001$). As protein assimilation may be affected by gastrointestinal surgery, patients who did not undergo surgery were analysed separately. The mean % dose ^{13}C cum

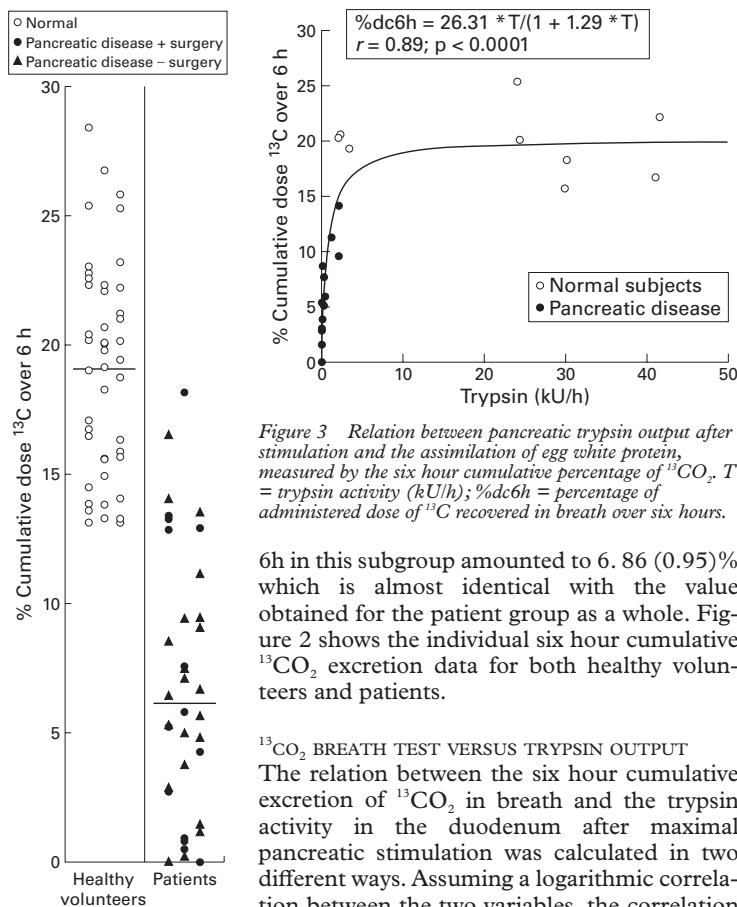Figure 2 Individual values for six hour cumulative percentage of $^{13}CO_2$ after ingestion of 22 g ^{13}C-labelled egg white protein by normal subjects (n = 48), patients with pancreatic disease who underwent gastrointestinal surgery (n = 9), and patients with pancreatic disease who did not undergo gastrointestinal surgery (n = 21).
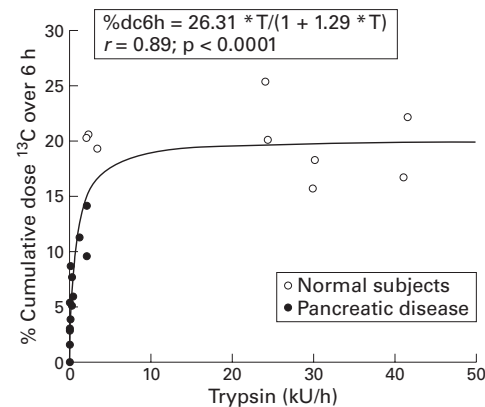

Figure 3 Relation between pancreatic trypsin output after stimulation and the assimilation of egg white protein, measured by the six hour cumulative percentage of $^{13}CO_2$. T = trypsin activity (kU/h); %dc6h = percentage of administered dose of ^{13}C recovered in breath over six hours.

6h in this subgroup amounted to 6. 86 (0.95)% which is almost identical with the value obtained for the patient group as a whole. Figure 2 shows the individual six hour cumulative $^{13}CO_2$ excretion data for both healthy volunteers and patients.

$^{13}CO_2$ BREATH TEST VERSUS TRYPSIN OUTPUT
The relation between the six hour cumulative excretion of $^{13}CO_2$ in breath and the trypsin activity in the duodenum after maximal pancreatic stimulation was calculated in two different ways. Assuming a logarithmic correlation between the two variables, the correlation coefficient of the test was found to be 0.88 (p<0.0001). If it is assumed that the relation between the cumulative excretion of $^{13}CO_2$ at six hours (% dose ^{13}C cum 6 h) and the trypsin activity is linear at low values and follows zero order (saturation) kinetics at high trypsin outputs, the mathematical expression of this relation can be written as follows:

$$\% \text{ cumulative 6 h} = \frac{A \times \text{trypsin}}{1 + B \times \text{trypsin}}$$

Non-linear regression analysis was performed on the data to estimate the constants A and B, using a home-made computer program written in Excel 5.0 for a 80486T computer (G Mys, personal communication). As the formula yielded a good fit (r = 0.89; p<0.0001)
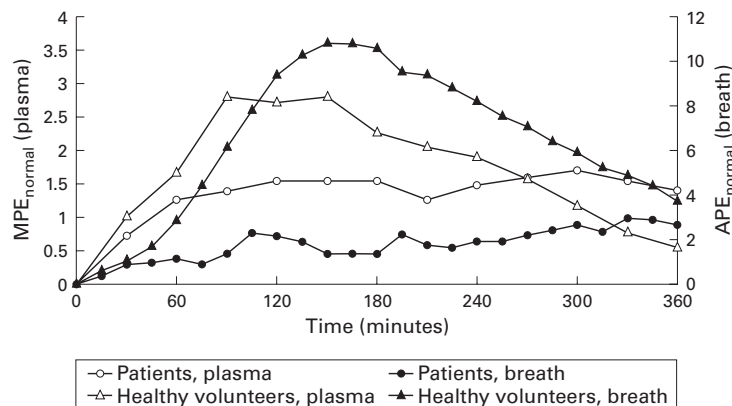

Figure 4 Recovery of $^{13}CO_2$ and L-[1-^{13}C]leucine in breath and plasma after ingestion of an egg protein meal labelled with L-[1-^{13}C]leucine in healthy volunteers (n = 6) and patients with pancreatic disease (n = 2).

(fig 3), the assumption that the relation between $^{13}CO_2$ excretion and trypsin activity follows saturation kinetics seems to be valid.

L-[1-^{13}C]LEUCINE CONTENT OF PLASMA VERSUS $^{13}CO_2$ CONTENT OF BREATH
Figure 4 shows the mean ^{13}C enrichment of breath and the mean L-[1-^{13}C]leucine enrichment of plasma after ingestion of a ^{13}C-labelled protein meal. Differences between healthy volunteers and patients are reflected in a similar way in the pattern of enrichment of breath and plasma. No significant difference was found in the area under the curve of L-[1-^{13}C]leucine enrichment, calculated by the trapezoidal rule, between patients and controls (8.52 v 10.65 respectively). A positive, although not significant, correlation (r = 0.52) was found between ^{13}C-breath test results and L-[1-^{13}C]leucine enrichment in plasma.

Discussion
Information about the clinical relevance of pancreatic dysfunction on the efficiency of protein assimilation in humans is scarce. The few studies attempting to elucidate this topic have relied on faecal nitrogen balance studies.[19][20] Nevertheless, balance studies take into account neither the nitrogen absorbed in the colon (which leads to an overestimation of digestive capacity) nor the endogenous secretion (which leads to an underestimation). The results of nitrogen balance studies may therefore be questioned. Protein assimilation can be evaluated more accurately in healthy volunteers using intubation techniques.[8][9] The widespread application of this approach is, however, prevented by its invasive character. Intubation moreover disturbs normal gastrointestinal physiology.[10]

In the past, breath tests have been used to study lipid[21][22] and carbohydrate[23] assimilation in a dynamic and non-invasive way. The recent availability of egg protein highly enriched with L-[1-^{13}C]leucine allowed breath test technology to be adapted for the study of protein assimilation as well.[13] The test meal consists of 22 g egg white protein, which represents a physiological load.

In this study, the ^{13}C-egg white breath test was used to evaluate the trypsin activity in the small intestine of healthy volunteers and patients with well documented pancreatic disease. A very good correlation is found between the $^{13}CO_2$ production and the trypsin output in the duodenum after maximal stimulation of the pancreas.

Direct pancreatic function tests such as the marker corrected perfusion technique used in this study have the highest sensitivity and specificity for the detection of exocrine pancreatic insufficiency and remain the yardstick for testing pancreatic function. Direct pancreatic function tests, however, have various practical disadvantages: they are time consuming, invasive, uncomfortable, not standardised, and require fluoroscopic tube placement. Therefore these direct tests are less suitable for routine application. Because of the good correlation between the $^{13}CO_2$ production and the

trypsin output in the duodenum, the ^{13}C-egg white breath test is promising both for the detection and follow up of exocrine pancreatic dysfunction.

Consequently, the ^{13}C-egg white breath test was performed on a large cohort of healthy volunteers and patients with well documented pancreatic disease. A significant impairment of the $^{13}CO_2$ excretion is shown in these patients after ingestion of the labelled protein test meal as compared with healthy subjects. Assuming no differences in gastrointestinal transit, metabolism of leucine after absorption, or the level of CO_2 production between healthy subjects and patients with pancreatic disease, this finding indicates significantly impaired protein assimilation in the patient group compared with the healthy volunteers. At present, data on leucine metabolism in pancreatic disease as well as in protein malabsorption syndromes are lacking. Pancreatic disease was complicated by diabetes mellitus in a subgroup of patients. As leucine oxidation has been reported by other researchers to be increased rather than impaired in diabetes mellitus,[24] concomitant diabetes cannot account for the decreased $^{13}CO_2$ excretion observed in the patient group. Because pancreatic disease was caused by excessive alcohol consumption in most patients, subclinical hepatic disease has to be considered as well. However, the influence of hepatic disease on leucine oxidation remains a controversial issue.[26] The breath test data in this study are in agreement with the generally accepted statement that intraluminal pancreatic digestion is very important in the overall process of protein assimilation.[15] Other mechanisms possibly contributing to deficient protein assimilation in pancreatic disease, such as accelerated small intestinal transit or inadequate absorption,[27 28] should be kept in mind as well.

Simultaneous measurement of ^{13}C enrichment of breath and enrichment of plasma L-[1-^{13}C]leucine after ingestion of a ^{13}C-labelled protein meal confirmed that assimilation kinetics are accurately reflected by the breath test. In other words, the rate of protein assimilation has to be considered the rate limiting step and not the metabolism of L-[1-^{13}C]leucine after absorption until exhalation of $^{13}CO_2$. It is obvious that the differences in L-[1-^{13}C]leucine enrichment between patients and healthy controls are less pronounced than the differences in $^{13}CO_2$ enrichment. Whether this can be attributed to an altered protein metabolism in patients with pancreatic disease will have to be elucidated by further research. No significant difference was found in the area under the curve of L-[1-^{13}C]leucine enrichment between patients and controls. A positive, although not significant, correlation was found between ^{13}C-breath test results and L-[1-^{13}C]leucine enrichment in plasma. The lack of significance is most probably due to the small sample size (six healthy volunteers and two patients) and the fact that protein assimilation in one of the two patients was only moderately affected (being delayed, rather than impaired).

Further studies are required to establish the sensitivity and specificity of the ^{13}C-egg white breath test in a clinically relevant population including patients with other gastrointestinal diseases such as coeliac sprue, radioenteritis, and short bowel syndrome. Motility disorders such as delayed gastric emptying may result in a lower tracer recovery over six hours in breath and therefore be responsible for false low values.

In conclusion, we showed an excellent correlation between the six hour cumulative $^{13}CO_2$ excretion in breath after ingestion of ^{13}C-labelled egg white protein meal and the duodenal trypsin output after maximal pancreatic stimulation. Consequently, the ^{13}C-egg white breath test can be considered a promising test for the evaluation and follow up of exocrine pancreatic function. Further studies are, however, required to establish the exact clinical usefulness of the ^{13}C-egg white breath test. The sensitivity and specificity of the test may be increased by simultaneous analysis of breath and serum for CO_2 enrichment and leucine concentration and enrichment respectively.

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